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Comparitive toxicity of cyclic peptides and depsipeptides in cultured rat hepatocytes

K.A. Mereish, R. Solow, Y. Singh[1] and R. Bhatnager[2]

Pathophysiology Division,[1] Bacteriology Division and [2]Pathology Division, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland 21701-5011, USA

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Introduction: Low-molecular-weight (MW), cyclic peptides (peptides linked through an amide linkage) and depsipeptides (peptides linked through an ester linkage) comprise a small group of metabolites produced by fungi, algae or bacteria. Among these cyclic peptides are cyclosporine (MW 1203), gramicidin-S (MW 1141) and microcystin-LR (MW 994), while valinomycin (MW 1111) and enniatin-B (MW 639) represent cyclic depsipeptides. These cyclic compounds possess varied pharmacological properties, ranging from antimicrobial activity [1] (valinomycin, enniatin-B, gramicidin-S, microcystin-LR) and strong immunosuppressive activity [2] (cyclosporine), to antimalarial activity [3] (valinomycin, cyclosporine, gramicidin). Many of these small cyclic peptides possess ionophoric properties, exhibiting differences in ion selectivity and affinities [1].

The toxicity (LD_{50}) of these compounds is in the range of microgram (microcystin-LR, 56 $\mu g\ kg^{-1}$, i.p., mice) [4], to milligram (cyclosporine, 107 $mg\ kg^{-1}$, i.v., mice) [2] quantities. Although mice treated with 200 $mg\ kg^{-1}$ day of cyclosporine [5], or sublethal doses of microcystin-LR [6], developed hepatic vascular congestion and fatty liver, there is no information available on the hepatotoxicity of the other cyclic peptides and depsipeptides. Microcystin-LR induces liver damage in mice [6] and necrosis of cultured hepatocytes after several hours of incubation with the toxin [7].

This study was designed to compare cell injury induced by these cyclic peptides and depsipeptides using the release of LDH and [^{14}C] adenine nucleotides [7] from cultured hepatocytes.

Material and methods: The following materials were obtained commercially from the indicated sources: gramicidin-S (Chemical Dynamics Corp., South Field, NJ), valinomycin (Calbiochem, La Jolla, CA), [^{14}C]adenine (50 mCi mmol^{-1}, New England Nuclear, Boston, MA), tissue culture medium and foetal bovine serum albumin (GIBCO, Grand Island, NY), tissue culture ware (Becton-Dickinson Labware, Lincoln Park, NJ), rat tail collagen, collagenase type IV, 5'-adenosine monophosphate (AMP), 5'-adenosine diphosphate (ADP), 5'-adenosine triphosphate (ATP), 5'-inosine monophosphate (IMP), adenosine, and adenine (Sigma, St. Louis, MO).

Male FW.LEW, congenic, inbred rats (G. Anderson, USAMRIID, Fort Detrick, Frederick, MD) weighing between 250-300 g were used for all experiments. Microcystin-LR (> 95% purity by HPLC) was obtained from Dr W. Carmichael, Wright State University, Dayton, Ohio. The following materials were gifts from the indicated sources: cyclosporine (Sandoz Laboratories, East Hanover, NJ, and enniatin-B (> 95% purity by TLC) from Dr H.R. Burmeister, Northern Regional Research Center, USDA, Peoria, IL.

Rat hepatocytes were isolated and cultured according to the method of Elliget and Kolaja [8]. After overnight incubation, hepatocytes were labelled with [^{14}C]adenine (0.2 μCi, 4 μM) as described by Shirhatti and Krishna [7]. Aliquots from the supernatants of prelabeled cells incubated with the cyclic peptides were analysed for the release of labelled-nucleotides and LDH at selected time intervals. The cells were lysed with 1 mL of 0.05% digitonin and an aliquat of the cell lysate was analysed for radioactivity (Beckman scintillation counter, model LS800, Fullerton, CA), LDH and protein content (Pierce protein reagent, Rockford, IL).

[^{14}C]-Adenine 5'-nucleotides (AMP, ADP, ATP, IMP), adenine and adenosine of hepatocyte supernatants were determined by thin layer chromatography on PEI-cellulose plates as described by Bochner and Ames [9] and compared with standards. The regions corresponding to the chromatographed standards were scraped from the plate and counted for radioactivity. Control and treated hepatocytes were examined under phase contrast microscope (Nikon Diaphot inverted phase contrast microscope) for morphological changes.

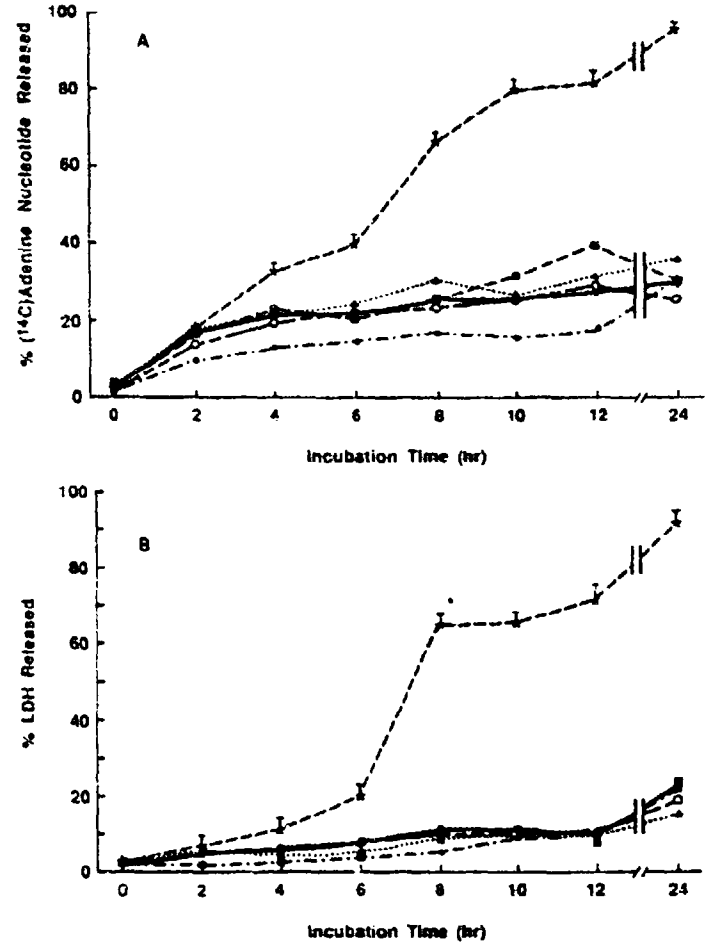
In conducting the research described in this report, the authors adhered to the *Guide for Laboratory Animal Facilities and Care* as promulgated by the Committee on the Guide for Laboratory Animal Resources, NAS/NRC.

Results and discussion: Hepatocytes incubated with 1 μM (or 10 μM, data not shown) of valinomycin, gramicidin-S, enniatin-B or cyclosporine over a 24 hour incubation period did not release [^{14}C]adenine nucleotides or LDH (Figures 1A and 1B) greater than control levels or induce any morphological changes (data not shown). However, hepatocytes treated with 1 μM microcystin-LR released significant amounts of labelled-nucleotide and LDH (Figures 1A and 1B) over time as compared to the release by control cells.

Furthermore, microcystin-LR (0.1-50 μM) induced dose- and time-dependent release of [^{14}C]adenine nucleotides (Figure 2) and LDH (data not shown). In addition, 1 μM microcystin-LR caused deformation (rounding and blebbing) in cell morphology (data not shown) consistant with the observation of an earlier report [10].

In order to determine whether the other cyclic peptides and depsipeptides induced toxicity in cultured rat hepatocytes at levels > 10 μM, cells were incubated with 50 μM of valinomycin, cyclosporine, or gramicidin-S for a total of 24 h. Enniatin-B was not tested at 50 μM due to inadequate supplies. At 50 μM, valinomycin, cyclosporine and gramicidin-S induced a significant time-dependent release of both labelled-nucleotides and LDH from hepatocytes as compared to control cells (Figures 3A and 3B).

Differences in the percent of marker release were observed in cells treated with cyclic peptides and depsipeptide. Gramicidin-S, microcystin-LR or valinomycin treated cells released approximately 80% of the total nucleotides (Figure 3A) within the first 2 h of incubation. Cyclosporine induced 90% release of labeled nucleotides from hepatocytes between 4-6 h of incubation (Figure 3A). The rate of LDH release



Figures 1A and 1B: Effect of 1 μM microcystin-LR (★—★), cyclosporine (○—○), gramicidin-S (●—●), valinomycin (■—■), or enniatin-B (▲—▲) on the release of [^{14}C]adenine nucleotides (1A) and LDH (1B) from cultured rat hepatocytes as compared to control (▼—▼). Each point represents the mean of six determinations, with 5–7% deviation. Values for the release of markers differed significantly from control figures only for microcystin-LR treatment after 4 h ($p < 0.05$, Student's t-test) Standard deviation bars were eliminated for clarity.

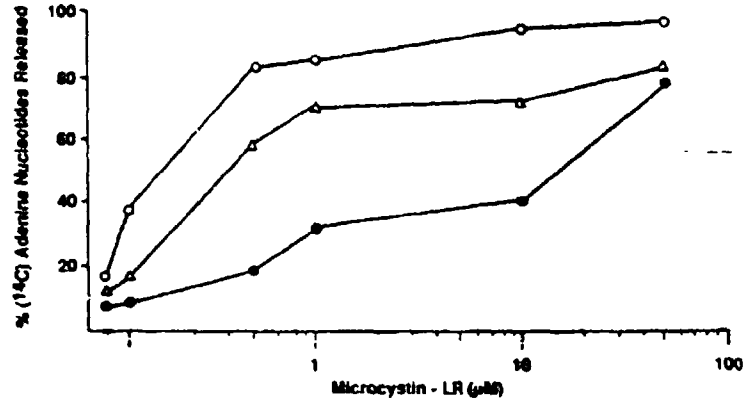
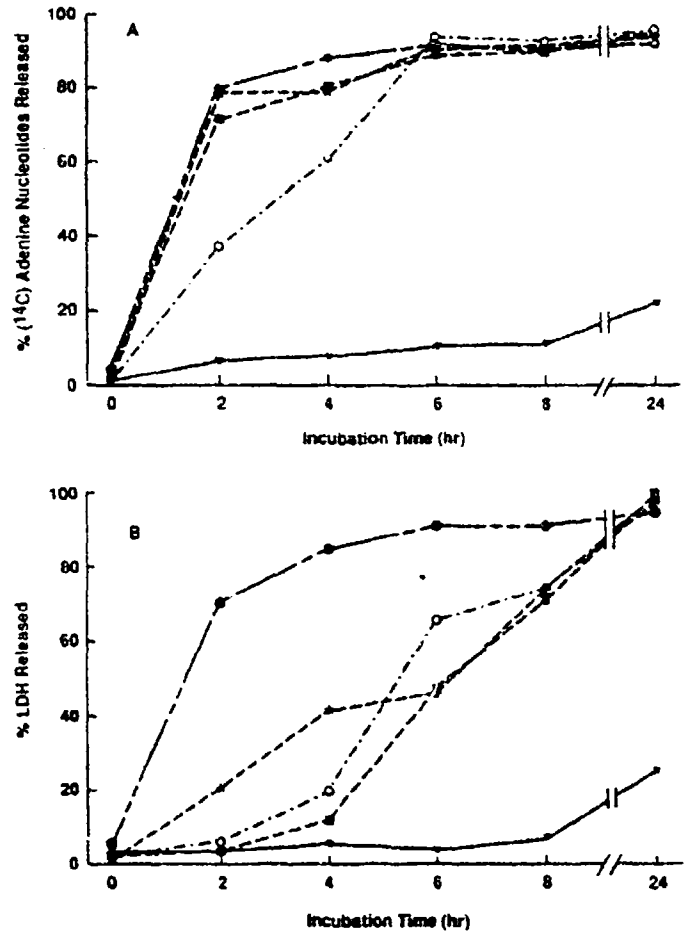


Figure 2: Effect of microcystin-LR (0.1 – 50 μM) on the release of [^{14}C]adenine nucleotides from cultured rat hepatocytes incubated for 2 (●—●), 4 (▲—▲), or 6 h (○—○). Each point represents the mean of three determinations, with 2–5% deviation. Standard deviation bars were eliminated for clarity.



Figures 3A and 3B: Effect of 50 μM microcystin-LR (★—★), cyclosporine (○—○), gramicidin-S (●—●), or valinomycin (■—■) on the release of [^{14}C]adenine nucleotides (3A) and LDH (3B) from cultured rat hepatocytes as compared to control (▼—▼). Each point represents the mean of six determinations, with ±7% deviation. Values for the release of markers differed significantly ($p < 0.05$, Student's t- test) from control figures for all treatments except for LDH release at 2 h in valinomycin and cyclosporine treatment. Standard deviation bars were eliminated for clarity.

from gramicidin-S treated cells was parallel to the release of labelled nucleotides (Figures 3A and 3B).

There was a 2 h lag in the release of LDH as compared to the release of nucleotides from cells treated with valinomycin or with cyclosporine. The percentages of LDH released at 2 and 4 h from cells treated with microcystin-LR, valinomycin and cyclosporine were significantly less than the percentages of nucleotides released at the same time points (Figures 3A and 3B).

The Rf values for AMP, ADP, ATP, IMP and adenosine were 0.68, 0.34, 0.1, 0.58 and 0.54, respectively. Due to the poor resolution in separating adenosine from IMP, the bands corresponding to these two compounds were quantified as one and reported as IMP. The distribution of labelled nucleotides released into the medium (8 h, 50 μM) was the same in control and toxin treated cells (AMP, 89%; ADP, 8%; ATP, 0.5%).

In conclusion, the release of LDH and adenine nucleotides from cultured rat hepatocytes indicated that at 50 μM, the cyclic peptides and depsipeptides tested in this study were hepatotoxic. Comparatively, at low concentration (1 μM), microcystin-LR exhibited the greatest cytotoxicity among these hepatotoxins.

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Reprint requests to: Dr. K.A. Meresh, Pathophysiology Division, U.S Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland 21701-5011, USA.

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